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Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications

(ribosomal proteins/radioimmunoassay/fluorescent antibody assay/peroxidase-conjugated antibody/autoradiography)

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ABSTRACT A method has been devised for the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. The method results in quantitative transfer of ribosomal proteins from gels containing urea. For sodium dodecyl sulfate gels, the original band pattern was obtained with no loss of resolution, but the transfer was not quantitative. The method allows detection of proteins by autoradiography and is simpler than conventional procedures. The immobilized proteins were detectable by immunological procedures. All additional binding capacity on the nitrocellulose was blocked with excess protein; then a specific antibody was bound and, finally, a second antibody directed against the first antibody. The second antibody was either radioactively labeled or conjugated to fluorescein or to peroxidase. The specific protein was then detected by either autoradiography, under UV light, or by the peroxidase reaction product, respectively. In the latter case, as little as 100 pg of protein was clearly detectable. It is anticipated that the procedure will be applicable to analysis of a wide variety of proteins with specific reactions or ligands.

Polyacrylamide gel electrophoresis has become a standard tool in every laboratory in which proteins are analyzed and purified. Most frequently, the amount and location of the protein are of interest and staining is then sufficient. However, it may also be important to correlate an activity of a protein with a particular band on the gel. Enzymatic and binding activities can sometimes be detected *in situ* by letting substrates or ligands diffuse into the gel (1, 2). In immunoelectrophoresis, the antigen is allowed to diffuse (3) or electrophoretically move (4) against antibody. A precipitate is then formed where the antigen and antibody interact. Modifications have been described in which the antigen is precipitated by directly soaking the separation matrix in antiserum (5, 6). The range of gel electrophoretic separation systems is limited by the pore size of the gels and diffusion of the antibody. The systems are also dependent on concentration and type of antigen or antibody to give a physically immobile aggregate.

Analysis of cloned DNA has been revolutionized (7) by the ability to fractionate the DNA electrophoretically in polyacrylamide/agarose gels first and then to obtain a faithful replica of the original gel pattern by blotting the DNA onto a sheet of nitrocellulose on which it is immobilized. The immobilized DNA can then be analyzed by *in situ* hybridization. The power of immobilized two-dimensional arrays has been extended to the analysis of proteins by use of antibody-coated plastic sheets to pick up the corresponding antigen from colonies on agar plates (8). Sharon *et al.* (9) have used antigen-coated nitrocellulose sheets to pick up antibodies secreted by hybridoma clones growing in agar.

In this report we describe a procedure for the transfer of proteins from a polyacrylamide gel to a sheet of nitrocellulose in such a way that a faithful replica of the original gel pattern is obtained. A wide variety of analytical procedures can be applied to the immobilized protein. Thus, the extreme versatility of nitrocellulose binding assays can be combined with high-resolution polyacrylamide gel electrophoresis. The procedure brings to the analysis of proteins the power that the Southern (7) technique has brought to the analysis of DNA.

MATERIALS AND METHODS

Immunogens and Immunization Procedures. *Escherichia coli* ribosomal proteins L7 and L12 were extracted (10) from 50S subunits and purified as described (11) by ion-exchange chromatography on carboxymethyl- and DEAE-cellulose. Antibodies were raised in a goat by injecting 250 μg of protein emulsified with complete Freund's adjuvant intracutaneously distributed over several sites. *Bacillus pertussis* vaccine (1.5 ml of Bordet–Gengou vaccine, Schweizerisches Serum- und Impfinstitut, Bern, Switzerland) was given subcutaneously with every antigen injection. Booster injections of the same formulation were given on days 38, 79, and 110. The animal was bled on day 117.

Subunits from chicken liver ribosomes (12) were combined in equimolar amounts, and 200-μg aliquots were emulsified with 125 μl of complete Freund's adjuvant injected at one intraperitoneal and four subcutaneous sites into BALB/c mice. Booster injections of 400 μg of ribosomes in saline were given intraperitoneally on days 33, 57, 58, and 59. The animals were bled on day 71.

Electrophoretic Blotting Procedures. Proteins were first subjected to electrophoresis in the presence of urea either in two dimensions (12) or in one-dimensional slab gels corresponding to the second dimension of the same two-dimensional system. The proteins were then transferred to nitrocellulose sheets as follows. The physical assembly used is shown diagrammatically in Fig. 1. A sheet of nitrocellulose (0.45 μm pore size in roll form, Millipore) was briefly wetted with water and laid on a scouring pad (Scotch-Brite) which was supported by a stiff plastic grid (disposable micropipette tray, Medical Laboratory Automation, Inc., New York). The gel to be blotted was put on the nitrocellulose sheet and care was taken to remove all air bubbles. A second pad and plastic grid were added and rubber bands were strung around all layers. The gel was thus firmly and evenly pressed against the nitrocellulose sheet. The assembly was put into an electrophoretic destaining chamber with the nitrocellulose sheet facing the cathode. The chamber contained 0.7% acetic acid. A voltage gradient of 6 V/cm was applied for 1 hr.

For polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (13) instead of urea, the procedure was as de-

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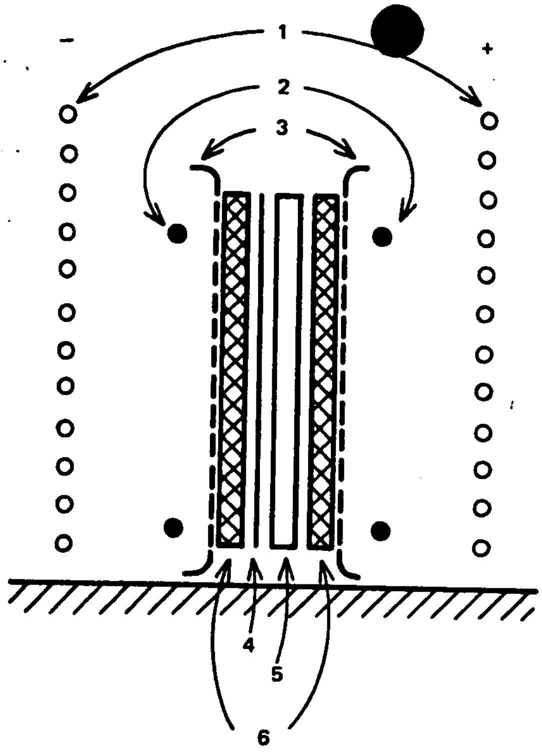

FIG. 1. Assembly for electrophoretic blotting procedure. 1, Electrodes of destainer; 2, elastic bands; 3, disposable pipette-tip tray; 4, nitrocellulose sheets; 5, polyacrylamide gel; 6, Scotch-Brite pads. Assembly parts are shown separated for visualization only.

scribed above except that the polarity of the electrodes was reversed and the electrode buffer was 25 mM Tris·192 mM glycine/20% (vol/vol) methanol at pH 8.3.

Staining for Protein. The blot may be stained with amido black (0.1% in 45% methanol/10% acetic acid) and destained with 90% methanol/2% acetic acid (see ref. 14).

Immunological Detection of Proteins on Nitrocellulose. The electrophoretic blots (usually not stained with amido black) were soaked in 3% bovine serum albumin in saline (0.9% NaCl/10 mM Tris·HCl, pH 7.4) for 1 hr at 40°C to saturate additional protein binding sites. They were rinsed in saline and incubated with antiserum appropriately diluted into 3% bovine serum albumin in saline also containing carrier serum with concentration and species as indicated in the legends. The sheets were washed in saline (about five changes during 30 min, total) and incubated with the second (indicator) antibody directed against the immunoglobulins of the first antiserum. As indicator antibodies we used ^{125}I-labeled sheep anti-mouse IgG. This had been purified with affinity chromatography on Sepharose-immobilized myeloma proteins and labeled by a modified version of the chloramine T method in 0.5 ml with 0.5 mg of IgG and 1 mCi of Na^{125}I (1 Ci = 3.7 × 10^{10} becquerels) for 60 sec at room temperature. The specific activity was approximately 1.5 μCi/μg of IgG. ^{125}I-Labeled IgG was diluted to 10^6 cpm/ml in saline containing 3% bovine serum albumin and 10% goat serum, and 3 ml of this solution was used for a nitrocellulose sheet of 100 cm^2. Incubation was in the presence of 0.01% NaN$_3$ for 6 hr at room temperature. The electrophoretic blots were washed in saline (five changes during 30 min, total) and thoroughly dried with a hair dryer. The blots were exposed to Kodak X-Omat R film for 6 days.

Fluorescein- and horseradish peroxidase-conjugated rabbit anti-goat IgG (Nordic Laboratories, Tilburg, Netherlands) were reconstituted before use according to the manufacturer's instructions. Fluorescein-conjugated antibodies were used at 1:50 dilution in saline containing 3% bovine serum albumin and 10% rabbit serum. After incubation for 30 min at room temperature, the blots were washed as above and inspected or photographed with a Polaroid camera under long-wave UV light through a yellow filter.

Horseradish peroxidase-conjugated IgG preparations were used at 1:2000 dilution in saline containing 3% bovine serum albumin and 10% rabbit serum. The blots were incubated for 2 hr at room temperature and washed as described above. For the color reaction (15), the blots were soaked in a solution of 25 μg of *o*-dianisidine per ml/0.01% H$_2$O$_2$/10 mM Tris·HCl, pH 7.4. This was prepared freshly from stock solutions of 1% *o*-dianisidine (Fluka) in methanol and 0.30% H$_2$O$_2$. The reaction was terminated after 20–30 min by washing with water. The blots were dried between filter paper. Drying considerably reduced the background staining. The blots were stored protected from light.

RESULTS

Electrophoretic Transfer of Ribosomal Proteins from Polyacrylamide Gels to Nitrocellulose Sheets. Most proteins or complexes containing protein adsorb readily to nitrocellulose filters (16), whereas salts, many small molecules, and RNA are usually not retained. These binding properties are widely used for binding assays with nitrocellulose filters. We found that proteins were retained on these filters equally well when carried towards the filter in an electric field. If the electric field was perpendicular to a slab gel containing separated proteins (see Fig. 1), we obtained a replica of the protein pattern on the nitrocellulose sheet. This is demonstrated with ribosomal proteins from *E. coli*; a conventionally stained gel (Fig. 2*A*) and a stained electrophoretic blot of an identical gel (Fig. 2*B*) are shown. All ribosomal proteins from chicken liver and *E. coli* ribosomes detectable on two-dimensional gels could be seen on the electrophoretic blots produced from them. An example of a blot from a two-dimensional gel is given in Fig. 3. When the original polyacrylamide gel was stained after blotting, no protein could be detected. Thus, the blotting procedure removed all protein from the gel.

To establish whether the proteins removed from the gels were quantitatively deposited on the nitrocellulose sheet, we separated ^{3}H-labeled proteins from chicken liver 60S ribosomal subunit by two-dimensional electrophoresis and compared the radioactivity that could be recovered from the blot with that recovered directly from the gel (Table 1). Single proteins or groups of poorly separated proteins were cut out and radioactivity was measured after combustion of the samples. The results were within the variability inherent to two-dimensional analyses. Variations could be accounted for by variable transfer of proteins into the second dimension gel and the acuity with which spots can be cut out.

At loads exceeding the capacity of nitrocellulose, losses of protein occurred. Titration with radioactive ribosomal proteins under blotting conditions showed that at concentrations below 0.15 μg/mm^2 all protein was adsorbed. Overloading became apparent when a second sheet of nitrocellulose directly underneath the first one took up protein or when protein became visible on the cathodal surface of amido black-stained blots.

The conservation of resolution together with the high recovery of ribosomal proteins simplifies the procedure for autoradiography. The common procedure involving drying of polyacrylamide gels under heat and reduced pressure (19), which is tedious and time consuming, may be eliminated. Because the proteins become concentrated on a very thin layer, autoradiography from ^{14}C- and ^{35}S-labeled proteins should be highly efficient even without 2,5-diphenyloxazole impregnation

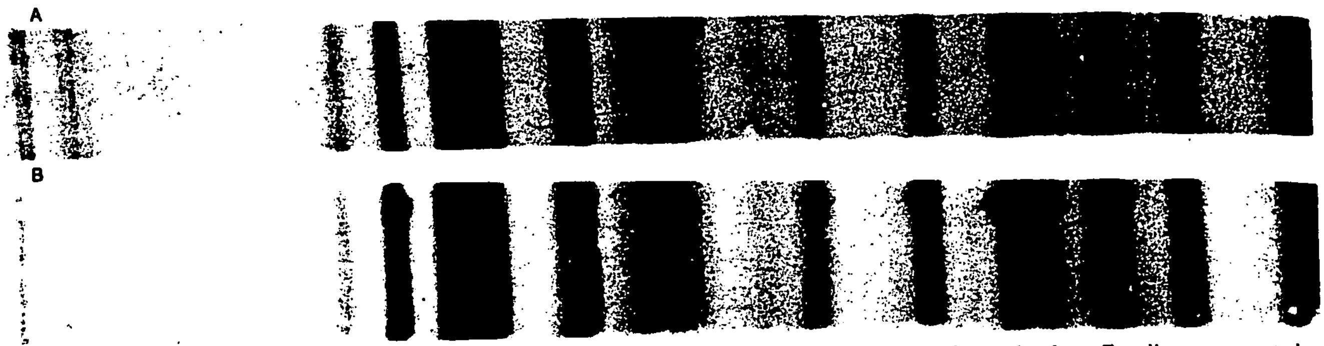

FIG. 2. Electrophoretic blotting of ribosomal proteins from one-dimensional gels. Total ribosomal proteins from *E. coli* were separated on an 18% polyacrylamide slab gel containing 8 M urea. (*A*) A section of the gel was stained with Coomassie blue; (*B*) another section was electrophoretically blotted and the blot was stained as described in *Materials and Methods*. Electrophoresis was from left to right.

(19). We have successfully obtained such autoradiograms from gels of ^{35}S-labeled proteins (not shown). Further, preliminary experiments with tritiated proteins have shown that dried blots may be processed for fluorography by brief soaking in 10% diphenyloxazole in ether (20).

The above experiments were done with ribosomal proteins separated on polyacrylamide gels containing urea. We have electrophoretically blotted proteins from sodium dodecyl sulfate by the modified procedure also described in *Materials and Methods*. Again, there was no loss of resolution. However, differences of staining intensities between proteins on the gel and the blot were apparent. In spite of the apparently incomplete recovery, blots from polyacrylamide gels containing sodium dodecyl sulfate may be used for detection of antigen in the same way as described below for ribosomal proteins (unpublished experiments).

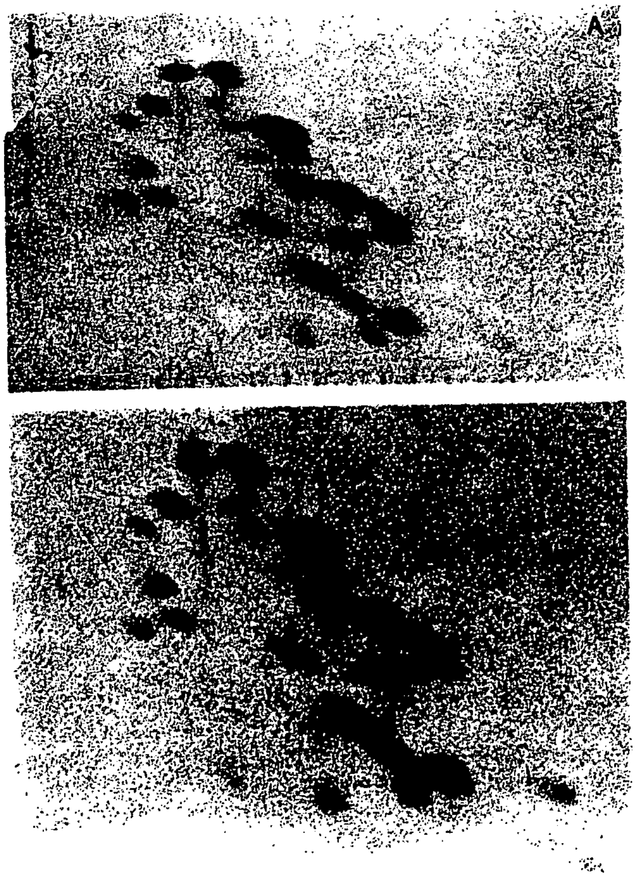

FIG. 3. Electrophoretic blotting of ribosomal proteins from two-dimensional gels. Proteins (35 μg) extracted from the 60S ribosomal subunit of chicken liver (12) were separated by two-dimensional gel electrophoresis. (*A*) Gel stained with Coomassie blue; (*B*) blot of an identical gel. Electrophoresis: 1st dimension, from left to right (towards cathode); 2nd dimension, top to bottom.

Detection of Antigen by Antibody Binding on Blots *In Situ*. We found that proteins transferred to nitrocellulose sheets remained there without being exchanged over several days. Because a blot could be saturated with bovine serum albumin to block the residual binding capacity of the sheet, it can be treated as a solid-phase immunoassay. In the following immunological applications, we used indirect techniques throughout. Thus, antibody bound by the immobilized antigen was detected by a second, labeled antibody directed against the first antibody, and in each case excess unbound antibody was washed out.

Table 1. Efficiency of transfer of ribosomal proteins to nitrocellulose sheets

Protein or group of proteins analyzed	Recovery on blot, %
3	123
4, 4A	104
5	111
6	107
7, 8	86
9	80
10	112
11	79
12, 16	93
13	95
15, 15A, 18	125
17	115
19	139
21, 23	118
26	114
27	143
28, 29	69
31	117
33	131

Ribosomal large-subunit proteins from chicken liver were tritiated by reductive methylation (17) and separated by two-dimensional electrophoresis (12) in the presence of 35 μg of carrier protein. Two identical gels were run. One was stained; the other was electrophoretically blotted on a nitrocellulose sheet. Spots were identified according to our nomenclature for chicken ribosomes (12), which differs only in minor respects from that established for rat ribosomes (18). Corresponding spots or groups of spots were cut from the gel and the blot. Their radioactivity was determined after conversion to tritiated water in a sample oxidizer (Oxymat).

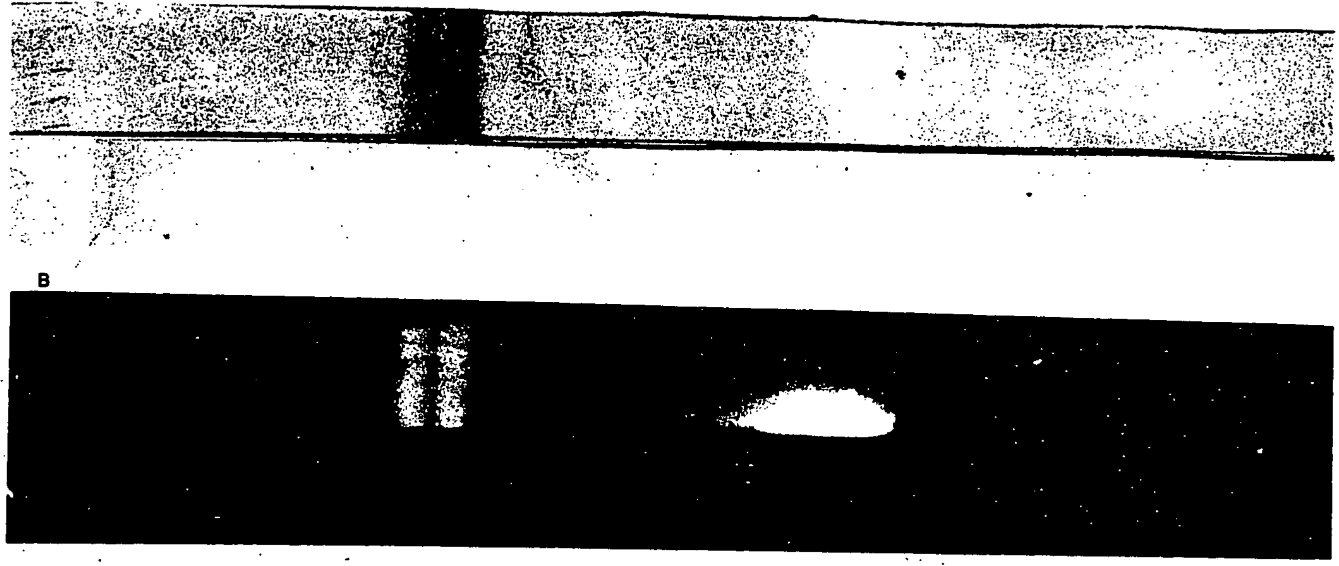

FIG. 4. Detection of *E. coli* ribosomal proteins L7 and L12 by (*A*) horseradish peroxidase- and (*B*) fluorescein-conjugated antibodies. Total ribosomal proteins from *E. coli* were separated and blotted as in Fig. 2. The anti-L7/L12 serum had a titer of 340 pmol of 70S ribosomes per ml of serum as determined by turbidity formation (20). Incubation was for 2 hr at room temperature in goat antiserum diluted 1:10 in saline containing 3% bovine serum albumin and 10% rabbit carrier serum and then with conjugated anti-goat IgG. In each case the lower strip is a control with preimmune antiserum. Electrophoresis was from left to right.

In Fig. 4 the detection of *E. coli* ribosomal proteins L7 and L12 with a goat serum specific for proteins L7 and L12 is shown: L7 is identical to L12, except for its *N*-acetylated NH_2-terminal amino acid (21). L7 and L12 fully crossreact immunologically (22) and are separated on acidic polyacrylamide gels (21). Both peroxidase- (Fig. 4*A*) and fluorescein-conjugated (Fig. 4*B*) antibodies were able to reveal immunoglobulin that was specifically retained by proteins L7 and L12. In each case, the lower gel is a control with preimmune serum. Peroxidase-conjugated antibodies were far more sensitive than fluorescein-conjugated ones. They could therefore be used at much higher dilution. This also permitted the detection of very small amounts of antigen. With a rabbit serum (23) we could detect 100 pg of L7 and L12 with serum and incubation conditions similar to those of the experiment described in Fig. 4 (not shown).

Because we can use the procedure to detect a specific antibody reacting with a specific protein after electrophoresis in polacrylamide, we should also be able to determine which proteins have elicited antibodies in a complex mixture of immunogens. In the experiment of Fig. 5, individual sera of five mice immunized with chicken liver ribosomes were tested. We used ^{125}I-labeled sheep anti-mouse immunoglobulins to detect the presence of mouse immunoglobulins. In all mice, antibodies were preferentially produced against slowly moving proteins, presumably of high molecular weight. The procedure can thus characterize the antigen population against which specific antibodies have been raised in a mixture of immunogens.

DISCUSSION

The electrophoretic blotting technique described here produces replicas of proteins separated on polyacrylamide gels with high fidelity. We obtained quantitative transfer with proteins from gels containing urea. This was established here with ribosomal proteins. More generally, nitrocellulose membranes have been used to retain proteins from dilute solutions for their subsequent quantitative determination (16). Still, there remains the possibility that certain classes of protein do not bind to nitrocellulose. In this case absorbent sheets other than nitrocellulose or different blotting conditions may be helpful.

We have demonstrated that proteins immobilized on nitrocellulose sheets can be used to detect their respective antibodies.

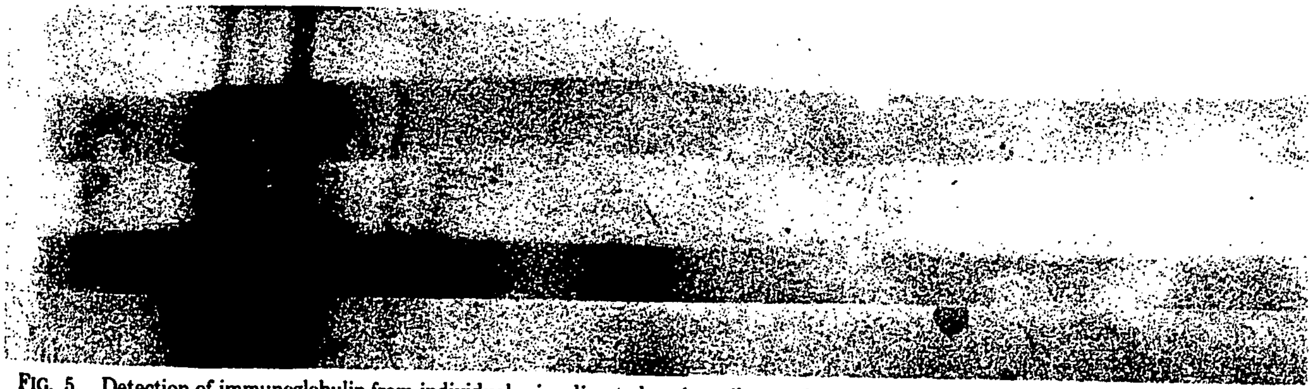

FIG. 5. Detection of immunoglobulin from individual mice directed against ribosomal proteins from chicken liver. Total protein from chicken liver ribosomes (12) was electrophoretically separated and blotted as in Fig. 2. Sera were obtained from five individual mice immunized against combined 40S and 60S subunits. The antisera were diluted 1:50 in saline containing 3% bovine serum albumin and 10% goat carrier serum. The blots were incubated in 250 μl of the diluted antiserum for 6 hr at room temperature. The blots were combined and treated with ^{125}I-labeled sheep anti-mouse IgG and autoradiographed. Electrophoresis was from left to right.

With radioactively labeled or peroxidase-conjugated antibodies the method is sensitive enough to detect small amounts of electrophoretically separated antigen, and this simple procedure can also be used to show the presence of small amounts of antibody in a serum of low titer. Because the antigen is immobilized on a sheet, the antibody is not required to form a precipitate with the antigen. The blotting technique therefore has the potential for immunoelectrophoretic analysis of proteins by using binding of Fab fragments or binding of antibodies against a single determinant, such as monoclonal antibodies produced by hybridomas (24). This could not be done by current immunoelectrophoretic techniques. If hybridoma clones are obtained from a mouse immunized with impure immunogen, it will be possible to use the technique to screen for clones making antibody directed against a desired antigen. Provided the desired antigen has a characteristic mobility in polyacrylamide gel electrophoresis, the appropriate clone can be selected without ever having pure antigen.

The procedure described here also has potential as a tool for screening pathological sera containing auto-antibodies—e.g., those against ribosomes (25–27). The precise identification of the immunogenic components may be a useful diagnostic tool for various pathological conditions.

A further advantage of immobilization of proteins on nitrocellulose is the ease of processing for autoradiography. Conventional staining, destaining, and drying of polyacrylamide gels takes many hours, and the exact drying conditions are extremely critical, especially for 18% gels as used in the second dimension for ribosomal proteins (12). When the proteins are transferred to a nitrocellulose support, as described here, the electrophoretic blotting takes 1 hr, staining and destaining less than 10 min, and drying an additional 5 min. This is thus both faster and simpler than conventional procedures, and it eliminates the tedious and hazardous procedure of soaking the gels in diphenyloxazole (19).

The technique has been developed to detect specific antisera against ribosomal proteins. However, it is applicable to any analytical procedure depending on formation of a protein–ligand complex. With the blotting technique, the usual procedure of forming a complex in solution and retaining it on a membrane would have to be reversed: the protein, already adsorbed to the membrane, would have to retain the ligand from a solution into which the membrane is immersed. Interactions that can possibly be analyzed in this way include hormone–receptor, cyclic AMP–receptor, and protein–nucleic acid interactions. The ligand may also be a protein. Enzymes separated on polyacrylamide gels could also be conveniently localized on blots by *in situ* assays. A critical requirement for these applications is that the protein is not damaged by the adsorption process and that binding sites remain accessible to ligands and substrates. In this respect, considerations similar to those in affinity chromatography and insoluble enzyme techniques pertain.

The method could also be adapted to the procedure of Cleveland *et al.* (28) for the analysis of proteins eluted from bands in polyacrylamide gels by one-dimensional fingerprints: one could label by iodination *in situ* on the nitrocellulose and then carry out the proteolytic digestion.

In preliminary experiments we have attempted to identify ribosomal RNA binding proteins by binding RNA to ribosomal proteins immobilized on nitrocellulose by the procedure of this paper, followed by staining for RNA (unpublished data), and have found a tendency for nonspecific binding. However, J. Steinberg, H. Weintraub, and U. K. Laemmli (personal communication) have independently developed a similar procedure for identifying DNA binding proteins.

We thank Drs. J. Schmidt and F. Dietrich for advice and help with immunization procedures and Mrs. M. Towbin for advice on setting up the peroxidase assay.

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